Recombinant human butyrylcholinesterase from milk of transgenic animals to protect against organophosphate poisoning

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Dangerous organophosphorus (OP) compounds have been used as insecticides in agriculture and in chemical warfare. Because exposure to OP could create a danger for humans in the future, butyrylcholinesterase (BChE) has been developed for prophylaxis to these chemicals. Because it is impractical to obtain sufficient quantities of plasma BChE to treat humans exposed to OP agents, the production of recombinant BChE (rBChE) in milk of transgenic animals was investigated. Transgenic mice and goats were generated with human BChE cDNA under control of the goat β-casein promoter. Milk from transgenic animals contained 0.1–5 g/liter of active rBChE. The plasma half-life of PEGylated, goat-derived, purified rBChE in guinea pigs was 7-fold longer than non-PEGylated dimers. The rBChE from transgenic mice was inhibited by nerve agents at a 1:1 molar ratio. Transgenic goats produced active rBChE in milk sufficient for prophylaxis of humans at risk for exposure to OP agents.

organophosphorus nerve agent | recombinant protein expression | transgenic production

Human plasma butyrylcholinesterase (huBChE) (EC 3.1.1.8) is a globular, tetrameric serine esterase with a molecular mass of ≈340 kDa that is stable in plasma with a half-life of ≈12 days (1, 2). Although the physiological function of huBChE is unclear, the enzyme prevents intoxication of animals exposed to organophosphorus (OP) compounds (3, 4). The huBChE enzyme also hydrolyzes many ester-containing drugs, such as cocaine and succinylcholine (5). The toxicity of OP agents is due to irreversible inhibition of acetylcholinesterase and the subsequent continuous stimulation of neurons by acetylcholine (6). Administration of exogenous huBChE, which irreversibly binds OP agents to prevent inactivation of acetylcholinesterase and continuous cholinergic stimulation, is a potential strategy for preventing toxicity from OP agents (4). Although huBChE has been obtained from human plasma by a large scale purification technique, this procedure is severely limited by the volume of human plasma needed (7). It is unlikely that a sufficient amount of enzyme could be purified commercially by this technique. Because of the 1:1 stoichiometry required for protection against exposure to OP agents (8), large quantities of huBChE are needed for effective prophylaxis and treatment of exposure. Compared with other potential enzymatic bioscavengers of OP agents, huBChE has a broad spectrum of activity, a relatively long half-life, and limited, if any, physiological side effects (9). Producing recombinant BChE (rBChE) is an alternative to purification of the enzyme from human plasma. Recombinant huBChE has been expressed in *Escherichia coli* (10), albeit in a nonfunctional form; mammalian 293T (11); and CHO (12) cells. However, these expression systems cannot economically produce sufficient quantities of rBChE with a residence time similar to native huBChE that would allow development of the enzyme as an agent for prophylaxis against OP poisoning.

The production of recombinant proteins by the mammary gland of transgenic animals is well established (13, 14). A variety of recombinant human proteins, including immunoglobins, growth hormone, and clotting factors have been expressed by the mammary gland and secreted in the milk of transgenic animals (13). This article describes the production of functional rBChE in the milk of transgenic mice and goats and the characterization of the recombinant protein. These studies illustrate the feasibility of producing large quantities of rBChE in transgenic animals for prophylaxis or treatment of humans exposed to OP agents.

Results

Generation of rBChE Transgenic Animals. A DNA expression vector, bCN-BChE, was developed to contain a 2.4-kb dimerized chicken β-globin gene insulator; a 6.7-kb goat β-casein gene promoter fragment, including the signal sequence in exon 2; a 1.7-kb human BChE cDNA fragment amplified from a huBChE cDNA clone [American Type Culture Collection (ATCC), Manassas, VA; catalog no. 65726]; and a 6.1-kb fragment consisting of the β-casein coding and 3′ noncoding regions (Fig. 1*A*). The plasmid backbone of bCN-BChE was removed by NotI digestion. The 16.9-kb rBChE transgene fragment was then gel-purified and microinjected into

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Abbreviations: BChE, butyrylcholinesterase; FVB, friend virus B-type; huBChE, human butyrylcholinesterase; OP, organophosphorus compounds; rBChE, recombinant butyrylcholinesterase; SEC, size exclusion chromatography.

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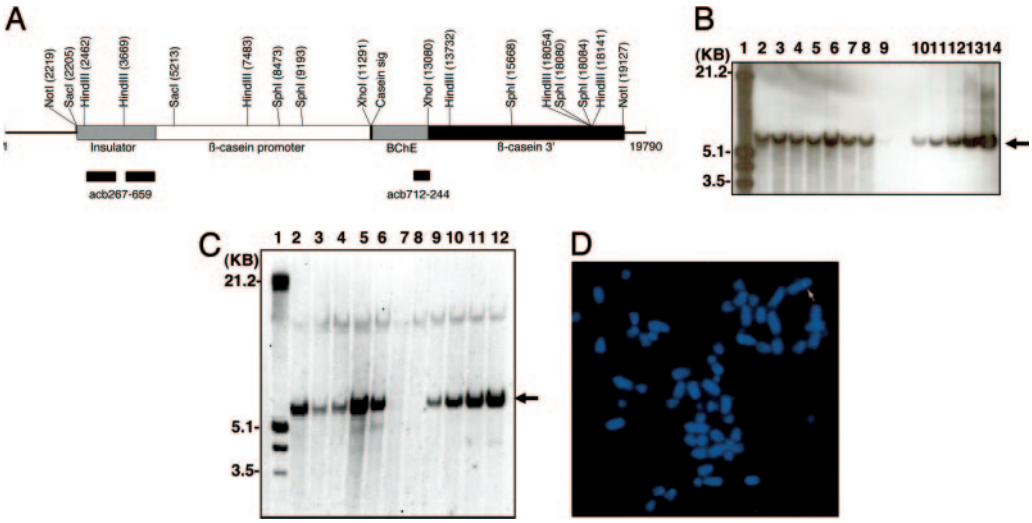

Fig. 1. Generation of rBChE transgenic animals. (*A*) Schematic of the bCN-BChE transgene used to generate the transgenic animals. The lower bars indicate the lengths of the PCR products and the primer pairs used for DIG-labeling of probes and screening transgenic animals. (*B*) Southern blot analysis of HindIII-digested genomic mouse DNA. Lane 1, DIG-labeled Marker III; lanes 2–8, genomic DNA from transgenic mice in the following order: 61-2B3F, 43-3A11F, 43-3A13F, 43-3A14F, 43-3A14A2F, 43-3A15F, and 43-3A15A9F; lane 9, genomic DNA from a nontransgenic FVB mouse; lanes 10–14, serial dilutions (2×, 4×, 8×, 16×, and 32×) of transgene plasmid DNA spiked into genomic DNA of a nontransgenic FVB mouse. The membrane was hybridized with Acb712-244. The arrow indicates the rBChE transgene (≈6.2 kb). (*C*) Southern blot analysis of SacI-SphI digested genomic goat DNA. Lane 1, DIG-labeled Marker III; lanes 2–7, genomic DNA from transgenic goats 1871F, 1876M, 1880F, 1900F, 1902F, and 1925F; lane 8, genomic DNA from a nontransgenic goat; lanes 9–12, serial dilutions (3×, 6×, 12×, and 24×) of transgene plasmid DNA spiked into genomic DNA of a nontransgenic goat. The membrane was hybridized with Acb712-244. The arrow indicates the rBChE transgene (≈6.5 kb). Endogenous BChE showed a weak band of ≈15 kb. (*D*) FISH analysis of metaphase spreads from a skin fibroblast cell sample of an F_2 transgenic goat, with an insulator probe. Arrow indicates localization of the rBChE transgene detected by FITC (shown in green) on a goat chromosome. Chromosomes were stained with DAPI (shown in blue).

the pronuclei of *in vitro*-produced zygotes to generate transgenic mice and goats. Four F_0 founder mice, two males and two females, and six F_0 founder goats, one male and five females, were generated. Southern blot and FISH analyses (Fig. 1 *B–D* and Table 1) revealed that the transgenic animals carried a variety of the rBChE transgene copies inserted randomly in their genomes. The transgene integrated in tandem head to tail or head to head arrays, as confirmed by Southern blot (data not shown). Two transgenic founder mice, 43-3M and 61-2F, and four transgenic founder goats, 1871F, 1876M, 1880F, and 1900F, bred with wild-type animals of the same strain transmitted the gene construct to their offspring. In addition, cloned copies of a selected female founder goat 1871F were generated by the somatic cell nuclear transfer technique (15). However, compared with the founder goat 1871F, reduced transgene copy numbers (10–12) and one integration site were detected from some of the cloned copies, as indicated by Southern blot and FISH analyses (Fig. 1*D* and Table 1), suggesting a mosaic expression pattern of the transgene in different cell populations in the founder goat. F_1 goats of the cloned copies of the founder goat were produced by a laparoscopic ovum pick-up-*in-vitro* fertilization technique (16). Initial herd expansion was performed in nontransgenic goats of New Zealand origin by artificial insemination from a master semen bank established from one of the male transgenic F_1 goats, 2219M, after it was established that this goat, along with other F_1 goats from the transgenic goat founder line, 1871F, also had 10–12 transgene copies integrated into a single chromosomal site in the genome (Table 1). Transgenic animals reported in this study were viable and healthy. Herd expansion of the rBChE transgenic goats is ongoing and is anticipated to produce all of the rBChE required for the preclinical and clinical studies evaluating rBChE for use in prophylaxis or treatment of OP intoxication. A herd of nontransgenic goats of New Zealand origin maintained within a biosecurity facility were the recipients of the artificial insemination program for generation of transgenic offspring that will constitute the commercial production herd. A total of 313 births have occurred with 615 live kids. Of 284 female kids, 121 were transgenic. Southern blot and FISH analyses revealed that most of the transgenic goats have 10–12 transgene copies and one integration site in the genomes, similar to the herd expansion originator goat 2219M.

Expression of rBChE in the Milk of Transgenic Animals. Milk samples from transgenic animals, collected after initiation of induced or natural lactation, were analyzed for the presence of the rBChE, using nondenaturing polyacrylamide gels stained for cholinesterase activity (17). The rBChE produced in the milk of the transgenic animals migrated as a mixture of dimer, tetramer and monomer, with dimer as the predominant form (Fig. 2*A*, lanes 2–4 for mouse milk, and Fig. 2*B*, lanes 3–5 for goat milk). Variation in the amount of the tetramer produced by the transgenic animals was also observed (Fig. 2*A*, lanes 2–4 and Fig. 2*B* lanes 3–5). The endogenous mouse BChE, detected from the milk of a low rBChE expression line, 44-2F (Fig. 2*A*, lane 4), and a negative control mouse (Fig. 2*A*, lane 5), migrated differently from the rBChE. Western blot analysis under denaturing and reducing conditions with a polyclonal anti-huBChE antibody confirmed the expression of the rBChE in the milk of the transgenic animals migrating at the expected size of the protein (≈90 kDa) (Fig. 2 *C* and *D*). The rBChE expressed in the milk of transgenic animals was a doublet of ≈90 kDa, which may represent different glycosylation states of the overexpressed protein. Other bands that could be identified on the Western blot with the same antibody in both the positive control (Fig. 2*D*, lane 1) and the milk samples (Fig. 2*D*, lanes 3–5) were a few minor bands with molecular masses >90 kDa, probably because of nonreduced forms of BChE. The identification of the oligomeric form of rBChE was based on migration relative to plasma huBChE. Plasma-derived huBChE (kindly provided by O. Lockridge, University of Nebraska, Omaha, NE) contained two bands, with the tetramer being the major species (Fig. 2 *A* and *B*, lane 1).

To gain further insight into the distribution of the oligomeric forms of the rBChE during lactation, raw milk samples from 12 F_2

Table 1. Summary of transgenic animals for rBChE and its expression in milk

Animal line	Sex	Copy no.	Integration site	rBChE, g/liter	Offspring	Generation	Sex	Copy number	Integration site	rBChE, g/liter
Mouse 43-3	M	8–10	ND	NA	3A11F	F_1	F	8–10	ND	0.3–0.6
					3A13F	F_1	F	8–10	ND	0.1–0.4
					3A14F	F_1	F	8–10	ND	0.2–0.6
					3A15F	F_1	F	8–10	ND	0.9–1.8
					3A14A2F	F_2	F	8–10	ND	0.1–0.8
					3A15A9F	F_2	F	8–10	ND	1–1.1
					3A14A2A2F	F_3	F	ND	ND	0.7–1.4
Mouse 43-5	M	8–10	ND	NA	—	—	—	—	—	—
Mouse 44-2	F	8–10	ND	0.02–0.05	—	—	—	—	—	—
Mouse 61-2	F	8–10	ND	0.2–0.6	2B3F	F_1	F	8–10	ND	0.1–0.5
Goat 1871	F	16–20	2–3	1–5	2114	Clone	F	10–12	1	ND
					2115	Clone	F	10–12	1	1–5
					2219	F_1	M	10–12	1	NA
					2220	F_1	F	10–12	1	1–5
					5719	F_2	F	10–12	1	1–5
Goat 1876	M	3–4	2	NA	—	—	—	—	—	—
Goat 1880	F	3–4	2–3	0.02–0.2	—	—	—	—	—	—
Goat 1900	F	30–35	1–2	0.5–3	—	—	—	—	—	—
Goat 1902	F	9–11	2–3	0.03–0.1	—	—	—	—	—	—
Goat 1925	F	2	1	BG	—	—	—	—	—	—

The concentrations for expression of rBChE, estimated by densitometry where 720 units = 1 mg of purified BChE were estimated from at least two independent assays. NA, not applicable; ND, not determined; BG, background; M, male; F, female; —, not available. Transgene copy numbers and integration sites of transgenic animals were determined by Southern blot and FISH analyses (see *SI Materials and Methods*). rBChE concentration (g/liter) in the milk of transgenic goats was determined by the Ellman assay.

transgenic goats were centrifuged and loaded into a size exclusion chromatography (SEC)-HPLC system. Collected fractions then were analyzed by the Ellman assay (18), and the oligomeric profile of the raw milk material was determined (Fig. 2*E*), confirming that the dimer is the major oligomeric form in the milk of the transgenic goats throughout the entire lactation period. SEC-HPLC combined with the Ellman assay, therefore, was a reliable and practical method to analyze the distribution of the oligomeric forms from the raw milk of transgenic goats.

Through herd expansion, kilogram quantities of rBChE have been produced in the milk of the transgenic female goats with >50 first lactations now completed. The rBChE concentration was consistently in the range of 1–5 g/liter throughout the entire lactation period, as assessed by the Ellman assay (Table 1). The rBChE expression data clearly demonstrate that rBChE is produced and secreted by the mammary gland of the transgenic animals. The purity of rBChE purified from the milk of transgenic goats was consistently >95%, as assessed by the silver-stained SDS/PAGE (Fig. 2*F*). The purified rBChE showed a similar 90-kDa doublet as described above. There were only minor differences in migration of huBChE purified from plasma and rBChE purified from the milk of transgenic goats, presumably because of differences in glycosylation between proteins from the two sources.

Pharmacokinetics of rBChE in Guinea Pigs. Once purified, the rBChE enzyme secreted in goat's milk had a short plasma half-life after either i.v. (Fig. 3*A*) or intramuscular injection (Fig. 3*B*) into guinea pigs. There was a significant improvement in the plasma half-life of the enzyme after modification with PEG (Fig. 3 *A* and *B*). PEGylation of the purified rBChE was performed with PEG 20,000, using lysine linkage chemistry. SDS/PAGE and SEC-HPLC/light scattering revealed that one to three PEGs were attached to each rBChE molecule (data not shown). Data from compartmental analysis provided estimates of pharmacokinetic parameters following i.v. and intramuscular administration of PEGylated and dimeric rBChE to male guinea pigs. The bioavailability of PEGylated rBChE was ≈46% with a plasma half-life of ≈44 h. By comparison, the bioavailability of unmodified dimer rBChE was ≈7.3% with a plasma half-life of ≈6.5 h (Table 2).

Activity of Expressed rBChE. In *in vitro* inhibition assays with the OP nerve agents soman (GD), sarin (GB), tabun (GA), or VX rBChE from transgenic mouse milk was inhibited at a molar ratio of ≈1:1 (Fig. 4). Similar inhibitory profiles were seen when purified rBChE from milk of transgenic goats was mixed with soman, sarin, tabun, or VX, using varying molar ratios of enzyme to nerve agent (8:1, 4:1, 1:1, and 1:4, respectively) (3).

The glycan components of the rBChE purified from goat's milk collected during early, middle, and later stages of a natural lactation were analyzed (Table 3). Compared with native BChE purified from human serum, the rBChE is underglycosylated, with more fucose and GalNac and less mannose, galactose, GlcNac, and sialic acid.

Discussion

Human BChE has a broad spectrum of efficacy against OP compounds used as nerve agents and is a promising bioscavenger for the treatment of humans exposed to these agents (4). Because the quantities of this enzyme that can be extracted and purified from human plasma are limited by the availability of out-dated human blood, recombinant DNA technology can play a major role in producing the quantities of enzyme needed for large scale use of this enzyme as an OP bioscavenger. To examine the feasibility of producing large quantities of rBChE in the milk of transgenic animals, rBChE was expressed first in transgenic mice as a proof-of-concept step, then in transgenic goats. By placing the rBChE gene under the control of the goat β-casein promoter, the rBChE protein can be produced in the milk of goats at concentrations of up to 5 g/liter. The ability to produce large quantities of rBChE in the milk of transgenic animals enables production of large quantities of active enzyme for prophylaxis or treatment of humans exposed to OP agents.

The tetrameric form of huBChE is retained in the circulation much longer than the rBChE produced in cell culture systems (19). The half life of the native enzyme in the circulation is influenced by

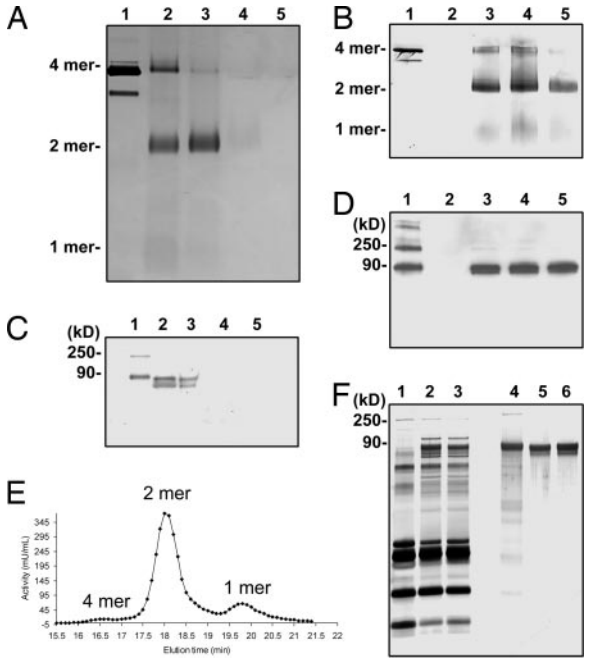

Fig. 2. rBChE expression, activity, and oligomeric forms in the milk of transgenic animals from natural lactations. For PAGE, 10 μl of samples were loaded onto each lane of a precast 4–20% Tris-glycine gel unless otherwise noted. (*A*) BChE activity gel staining of milk samples from transgenic mice. Lane 1, purified plasma huBChE (25 units/ml); lane 2, diluted 61-2B3F milk (1:50); lane 3, diluted 43-3A-15F milk (1:1,000); lane 4, diluted 44-2 milk (1:3); lane 5, diluted FVB mouse milk (1:3). (*B*) BChE activity gel staining of milk samples from transgenic goats. Lane 1, purified plasma huBChE (10 units/ml); lane 2, diluted milk from a nontransgenic goat (1:2); lanes 3–5, diluted milk containing rBChE from 3 transgenic goats: 1871F (1:80), 2220F (1:40), and 5719F (1:40). (*C*) Western blot analysis of milk from transgenic mice under denaturing and reducing conditions. Lane 1, purified plasma huBChE (25 units/ml); lane 2, diluted 61-2B3F milk (1:25); lane 3, diluted 43-3A-15F milk (1:500, 30 μl); lane 4, diluted 44-2 milk (1:1.5, 20 μl); lane 5, diluted FVB mouse milk (1:1.5). (*D*) Western blot analysis of milk from transgenic goats under denaturing and reducing conditions. Lane 1, purified plasma huBChE (10 units/ml); lane 2, diluted milk from a nontransgenic goat (1:2); lanes 3–5, diluted milk containing rBChE from 3 transgenic goats: 1871F (1:80), 2220F (1:40), and 5719F (1:40). (*E*) Analysis of milk from a transgenic goat by SEC-HPLC. Raw milk samples from 5719F, an F_2 goat of 2219M, were briefly centrifuged then loaded into a SEC-HPLC system. Fractions were collected over time and analyzed by the Ellman assay. The specific activity of the rBChE versus SEC-HPLC collection intervals was plotted. (*F*) Silver-stained SDS/PAGE on purified rBChE from the milk of transgenic goats. Lane 1, diluted milk from a nontransgenic goat (1:100); lane 2, diluted milk from the transgenic founder goat, 1871F (1:100); lane 3, diluted milk from the transgenic goat, 2220F (1:100); lane 4, purified plasma huBChE (10 units/ml); lane 5, purified rBChE from milk of the transgenic founder goat, 1871F (250 ng); lane 6, purified rBChE from milk of the transgenic goat, 2220F (250 ng).

the oligomerization of the enzyme and the heterogeneity in charge and size because of the surface glycans (20). Staining for rBChE on nondenaturing activity gels and analyses of the raw milk samples by SEC-HPLC indicate that the expressed enzyme is mainly in the dimeric form. By comparison, the BChE isolated from human plasma was predominately a tetramer. An *in vitro* expression cassette with the same rBChE cDNA used to generate the transgenic mice and goats was expressed in MAC-T, a bovine mammary gland epithelial cell line, with production of a predominately tetrameric form of the enzyme (A.L. and Y.-J.H., unpublished data). ColQ PRAD peptide, a proline-rich attachment domain encoded by the ColQ gene, is necessary for efficient assembly of the tetrameric forms of BChE and acetylcholinesterase *in vitro* and *in vivo* (11, 19, 21). It is possible that the higher level of expression of rBChE by mouse and goat mammary glands might have exceeded the capacity of the intracellular protein assembly machinery to

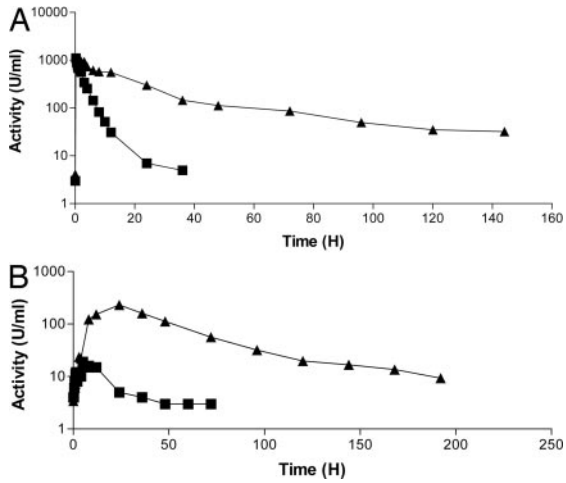

Fig. 3. Clearance of purified rBChE by guinea pigs. The guinea pigs were injected IV (*A*) or IM (*B*) with PEGylated rBChE (▲) or dimer rBChE (■). Residual BChE activity (units/ml), measured in blood by the Ellman assay, was plotted over time (hours).

efficiently assemble tetramers, such that they are a minor component of the total rBChE produced. There may not be enough ColQ PRAD peptide available in this expression system, thereby altering the oligomeric distribution of the rBChE secreted in the milk of transgenic animals.

Glycosylation is one of the most important posttranslational modifications of proteins (22). Glycans on tetrameric forms of human, equine, and fetal bovine plasma cholinesterases in serum contain predominantly complex bi-antennary glycan structures, which may increase the stability of these enzymes in the circulation (20). Mammary gland cells have the capacity to secrete proteins that are N- or O-glycosylated. There have been numerous recombinant proteins expressed and secreted in the milk of transgenic animals. Some of them, for example, α1-antitrypsin and superoxide dismutase, are glycosylated like their native counterparts (22). Others, such as human tissue plasminogen activator and human antithrombin III, are underglycosylated or not glycosylated in an appropriate manner (23). It is clear that the dimeric rBChE purified from goat's milk was underglycosylated, especially with sialic acid, as compared with BChE from human plasma. These differences in glycosylation of recombinant proteins may have more impact on *in vivo* stability than on biological activity (22).

PEGylation often improves the pharmacokinetic and toxicity profiles of recombinant proteins (24). Attachment of hydrophilic PEG molecules to a peptide or protein improves water solubility and forms a barrier to prevent enzyme degradation, renal clearance, interaction with cell surface proteins, or development of neutralizing antibodies (24). In this study, the significant increase in the plasma half-life of the PEGylated rBChE purified from the milk of transgenic goats and administered to guinea pigs compared with the native rBChE dimer suggests that, despite poor glycosylation of the recombinant protein, PEGylation is an effective strategy to improve the pharmacokinetic profile of this enzyme.

Table 2. Summary of the pharmacokinetic parameters of rBChE in guinea pigs following intravenous and intramuscular injections

Parameters	PEGylated rBChE	Dimer rBChE
Half-life i.v., h	44.2	6.5
Half-life i.m., h	40.7	7.1
Bioavailability, %	46	7.3

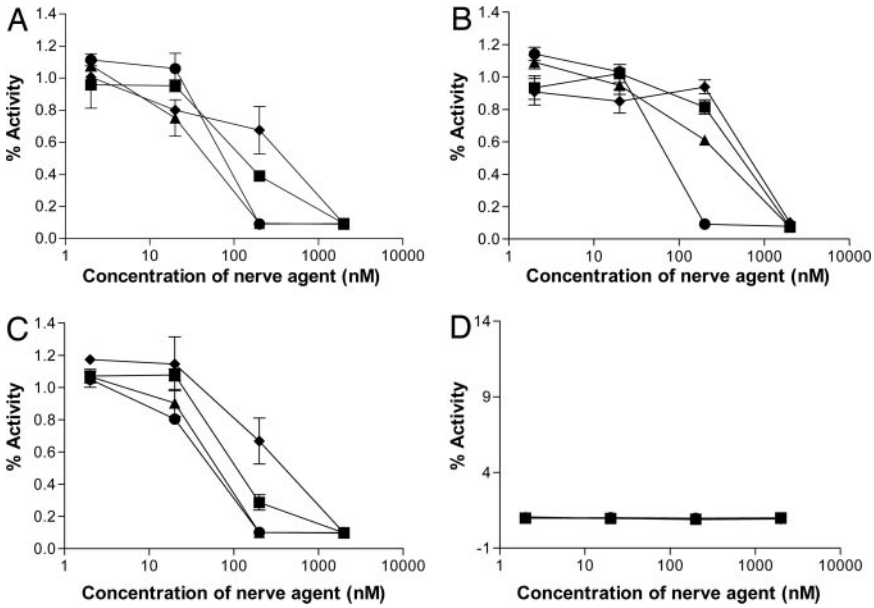

Fig. 4. *In vitro* inhibition by nerve agents of rBChE contained in the milk of transgenic mice. Binding was performed with diluted raw mouse milk samples from three transgenic mice [3A14F (*A*), 3A15F (*B*), and 2B3F (*C*)] and a control FVB mouse (*D*). The reactions were carried out in the presence of tabun (GA, ◆), sarin (GB, ■), soman (GD, ▲), and VX (●), respectively. Residual enzyme activity was measured by the Ellman assay. Data points represent the mean ± SD from duplicates in each mouse.

To successfully use the milk expression system in transgenic animals for manufacturing large quantities of rBChE, it is essential that the enzyme be functionally similar to huBChE. The administration of purified, PEGylated, rBChE from goat milk, in the absence of any other pretreatments or therapy, was found to protect guinea pigs against exposure to multiple lethal doses of the nerve agents VX or soman, with 100% survival and no signs of toxicity [L. A. Lumley, C. L. Robison, B. Somsamayuong, and D.M.C., unpublished data; please also see a review by Lenz *et al.* (4)]. The *in vivo* efficacy data in guinea pigs from the goat milk-derived and PEGylated rBChE as well as *in vitro* nerve agent binding and inhibition experiments with rBChE from the milk of transgenic mice or purified from the milk of transgenic goats indicated that the expressed enzyme was similar to huBChE in plasma with regard to activity against a variety of nerve agents, suggesting that the rBChE produced in the milk of transgenic goats will likely be a useful bioscavenger of OP nerve agents.

Transgenic animal technology has been used extensively in biomedical and agricultural research (25). The use of transgenic farm animals to produce pharmaceutically important recombinant proteins, such as antibodies, anti-clotting factors, and growth factors, in the mammary gland is well documented (26). Compared with other farm animal species, the goat is frequently used because of its relatively low cost of maintenance and faster breeding (27). The present study demonstrates the feasibility of large-scale production of rBChE in transgenic goats with kilogram quantities of functional recombinant huBChE produced in the milk of transgenic goats. The use of transgenic goats as bioreactors for the production of functional rBChE will provide a reliable source of this enzyme for prophylaxis or treatment of humans exposed to OP compounds used as agricultural or chemical warfare agents.

Table 3. Carbohydrate analysis of rBChE

Monosaccharide	rBChE derived from goat milk	Native huBChE*	rBChE from other expression systems*
Fucose	39	24	58
Mannose	291	428	165
Galactose	102	333	128
GalNac	42	20	19
GlcNac	303	613	238
Sialic acid	113	309	110

*Values reported in ref. 20. Values are in nanomoles per milligram.

Methods

Production of Founder and Subsequent Generation of Transgenic Animals. The rBChE transgene DNA expression cassette was constructed as described in supporting information (SI) *Materials and Methods*. Transgenic mice were produced and maintained at McIntyre Transgenic Core Facility of McGill University (Montreal, QC, Canada). Animal studies were carried out in accordance with guidelines on the care and use of experimental animals from the Canadian Council of Animal Care. Transgenic mice were generated in a friend virus B-type (FVB) background strain (Charles River Laboratories, Wilmington, MA) as described in ref. 28. The bCN-BChE expression vector containing the transgene was microinjected into fertilized eggs, and 22 pups were born. At 2–3 weeks of age, tail biopsies were taken under anesthesia, and DNA was prepared according to standard procedures (29). Transgenic founder mice were bred with wild-type mice of the same strain for the production of F_1, F_2, and F_3 generations.

The production and maintenance of transgenic goats were conducted at the PharmAthene Canada Caprine Production Farm. Animal studies were carried out according to protocols approved by the Animal Care Committee of PharmAthene Canada. The production of the founder goats and subsequent generation of rBChE transgenic goats was performed as described in ref. 30. Briefly, 3 μg/ml of the transgene DNA fragment were microinjected into *in vitro* produced goat zygotes. After a brief *in vitro* culture, the zygotes were transferred to recipient goats and pregnancies confirmed by transrectal ultrasonography. Newborn kids were separated from recipient goats at birth to prevent disease transmission. Transgenic founder goats were bred with wild-type goats of the same strain for the production of subsequent generations. Cloned copies of a selected female founder goat, 1871F, were generated by somatic cell nuclear transfer (15). F_1 goats of the cloned copies of the

founder goat were obtained by laparoscopic ovum pick-up-*in-vitro* fertilization as described in ref. 31. Herd expansion was performed in nontransgenic New Zealand herd goats by artificial insemination from a master semen bank established from one of the male transgenic F_1 goats, 2219M and some of its male offspring. Transgenic female goats were hormonally induced into lactation (32) at 2 months of age to measure expression of rBChE in the milk before natural lactation took place at 12 months age.

rBChE transgenic animals were identified and characterized by PCR, Southern blot and FISH analysis. The methods are described in *SI Materials and Methods*.

SEC-HPLC of rBChE in the Milk of Transgenic Goats. Raw milk samples collected from 12 F2 transgenic goats during natural lactation were centrifuged twice for 90 seconds each at room temperature at $9,300 \times g$, and the clear supernatants were diluted 40-fold with a HPLC mobile phase containing 20 mM NaP, 200 mM NaCl, and 0.04% NaN_3. The samples were then filtered through a syringe-driven 0.22-μm filter. A 10-μl aliquot of each sample was injected and analyzed on a SEC-HPLC system (Waters Alliance 2695 separations module; Waters, Milford, MA) with UV detection at 280 nm. The SEC column used was a Shodex (Kawasaki, Japan) KW-803 (8 mm $\times$ 300 mm with an exclusion limit of 1.7×10^5 and minimum of 21,000 theoretical plates). The Shodex protein KW-G guard column (6 mm $\times$ 50 mm) was used with the column. Separation of tetramer, dimer, and monomer peaks of the rBChE in the milk was obtained. Collected fractions were analyzed by the Ellman assay. Data were plotted as rBChE specific activity versus SEC-HPLC collection intervals. By using area counts, the percentages of tetramer, dimer, and monomer were determined.

PEGylation of Purified rBChE. rBChE activity assay and rBChE purification were performed as described in *SI Materials and Methods*. Purified rBChE (0.5 mg/ml) was mixed quickly with PEG 20,000 at a ratio of 1:80 in 50 mM sodium phosphate, pH 8.0 and incubated at 24°C for 2 h. The PEGylated proteins were then purified by affinity chromatography with a procainamide column as described above.

Pharmacokinetic Studies of rBChE. Pharmacokinetic studies in animals were carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted by the U.S. National Institutes of Health and were conducted by MDS Pharma Services (Laval, Quebec, Canada). Male Hartley guinea pigs (Charles River) were divided into three groups. Animals in Groups 1 and 2 received the test article by i.v. and intramuscular injection, respectively, whereas those in Group 3 received a vehicle control via intramuscular injection. The guinea pigs were injected with 72.3 mg/kg purified PEGylated rBChE (n = 14, 12, and 4 in Groups 1, 2, and 3, respectively) or 79.4 mg/kg purified rBChE dimers (n = 9, 12, and 12 in Groups 1, 2, and 3, respectively). Timed blood samples were collected from the animals for 192 h after dosing, and BChE activity was tested by the Ellman assay. The pharmacokinetic data were analyzed by a two-compartment model with NONMEM software, Version 5 (GloboMax, Ellicott City, MD) with data from Group 3 fit into the model as random variability.

***In Vitro* Inhibition of rBChE by OP Agents.** rBChE contained in the milk of transgenic mice was combined with different concentrations of the OP agents soman (GD), sarin (GB), tabun (GA), or VX and incubated at 25°C for 10 min, using the proper safety precautions. The BChE activity for each incubation was measured by the Ellman assay, and the results were expressed as a percentage of residual activity. Data were plotted and analyzed by using Microsoft Excel (Microsoft, Redmond, WA).

Carbohydrate Analysis of rBChE. Monosaccharide analysis of purified rBChE in dimer form from the milk of 2220F, a F_1 transgenic goat, was performed by GlycoSolutions (Worcester, MA).

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